Articles

Multiple Interactions of the Asp$^{2.61(98)}$ Side Chain of the Gonadotropin-Releasing Hormone Receptor Contribute Differentially to Ligand Interaction†

Colleen A. Flanagan,[‡,§,||] Vladimir Rodic,[||,⊥] Karel Konvicka,[#] Tony Yuen,[‡] Ling Chi,[⊥] Jean E. Rivier,[▽] Robert P. Millar,[▼,◇] Harel Weinstein,[#,◆] and Stuart C. Sealfon*[,‡,⊥]

Department of Neurology, Fishberg Research Center in Neurobiology, Department of Physiology and Biophysics, and Department of Pharmacology, Mount Sinai School of Medicine, New York, New York 10029, The Salk Institute, San Diego, California 92186, and Department of Chemical Pathology, University of Cape Town Medical School, Observatory, 7925, South Africa

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ABSTRACT: Mutation of Asp$^{2.61(98)}$ at the extracellular boundary of transmembrane helix 2 of the gonadotropin-releasing hormone (GnRH) receptor decreased the affinity for GnRH. Using site-directed mutagenesis, ligand modification, and computational modeling, different side chain interactions of Asp$^{2.61(98)}$ that contribute to high-affinity binding were investigated. The conservative Asp$^{2.61(98)}$Glu mutation markedly decreased the affinity for a series of GnRH analogues containing the native His^2 residue. This mutant showed smaller decreases in affinity for His^2-substituted ligands. The loss of preference for His^2-containing ligands in the mutant receptor shows that Asp$^{2.61(98)}$ determines the specificity for His^2. Analysis of the affinities of a series of position 2-substituted ligands suggests that a hydrogen bond forms between Asp$^{2.61(98)}$ and the δ NH group of His^2 and that Asp$^{2.61(98)}$ forms a second hydrogen bond with the ligand. Substitution of Asp$^{2.61(98)}$ with an uncharged residue further decreased the affinity for all ligands and also decreased receptor expression. Computational modeling indicates an intramolecular ionic interaction of Asp$^{2.61(98)}$ with Lys$^{3.32(121)}$ in transmembrane helix 3. The uncharged, Lys$^{3.32(121)}$Gln mutation also markedly decreased agonist affinity. The modeling and the similar phenotypes of mutants with uncharged substitutions for Asp$^{2.61(98)}$ or Lys$^{3.32(121)}$ are consistent with the presence of this helix 2−helix 3 interaction. These studies support a dual role for Asp$^{2.61(98)}$: formation of an interhelical interaction with Lys$^{3.32(121)}$ that contributes to the structure of the agonist binding pocket and an interaction with His^2 of GnRH that helps stabilize agonist complexing.

The decapeptide gonadotropin-releasing hormone (GnRH)[1] (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2) is the primary mediator of control of reproductive function by the nervous system. GnRH is released from the hypothalamus and interacts with receptors on the gonadotrope cells of the pituitary to stimulate release of luteinizing and follicle stimulating hormones, which regulate gonadal activity.

The heptahelical GnRH receptor is a member of the large rhodopsin-like family of G protein-coupled receptors (GPCRs) (*1*). Among GPCRs, the determinants of ligand binding have been most successfully elucidated for the biogenic amine receptors (*2*−*4*). The greater complexity and variability of peptide ligands have made determination of their binding interactions more difficult. Studies of ligand binding and activation of the GnRH receptor have identified several receptor residues which are important for high-affinity ligand binding (*1*). The Asn$^{2.65(102)}$ residue (see Experimental Procedures for residue numbering scheme) at the extracellular

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* Correspondence should be addressed to this author at the Department of Neurology, Box 1137, Mount Sinai School of Medicine, One Gustave Levy Place, New York, NY 10029. Phone: (212) 241-7075, Fax: (212) 348-1310, Email: sealfon@msvax.mssm.edu.

‡ Department of Neurology, Mount Sinai School of Medicine.

§ Current address: Department of Medical Biochemistry, University of Cape Town Medical School, Observatory, 7925, South Africa.

|| The first two authors contributed equally to this work.

⊥ Fishberg Research Center in Neurobiology, Mount Sinai School of Medicine.

Department of Physiology and Biophysics, Mount Sinai School of Medicine.

▽ The Salk Institute.

▼ Department of Chemical Pathology, University of Cape Town Medical School.

◇ Current address: Medical Research Council Reproductive Biology Unit, 37 Chalmers St., Edinburgh, EH3 9ET, United Kingdom.

◆ Department of Pharmacology, Mount Sinai School of Medicine.

[1] Abbreviations: GnRH, gonadotropin-releasing hormone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EC_{50}, the agonist concentration required for a half-maximal IP response; E_{max}, maximal IP response; GnRH II, [His^5,Trp^7,Tyr^8]-GnRH; GnRH-A, [D-Ala^6,Pro^{9}NHEt]-GnRH; GPCR, G protein-coupled receptor; IC_{50}, peptide concentration required for 50% inhibition of ^{125}I-Cetrorelix binding; IP, inositol phosphate(s); TMS, transmembrane segment.

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boundary of the second transmembrane segment (TMS) binds GnRH through a hydrogen bond with the carboxy-terminal $GlyNH_2$ moiety of the peptide (*5*). The $Lys^{3.32(121)}$ residue, in the same TMS 3 locus as the $Asp^{3.32}$ residue which is important for ligand binding in the biogenic amine receptors (*4*), has a role in the high-affinity binding of GnRH agonists, but not GnRH antagonists (*6*). $Glu^{7.32(301)}$ in the third extracellular loop of the mouse GnRH receptor is the determinant of high-affinity recognition of the positively charged Arg^8 side chain of GnRH (*7*).

Previous studies indicated that the acidic $Asp^{2.61(98)}$ residue at the extracellular end of TMS 2 is important for GnRH receptor function (*7*). In addition to its ability to form salt bridge interactions with positively charged functional groups, the carboxylate side chain of Asp has the capacity to simultaneously form multiple interactions with uncharged, polar hydrogen bond donor groups. Charged side chains within proteins generally fulfill all possible hydrogen bond interactions with neighboring groups or with water in the solvent (*8, 9*). This creates the potential for the $Asp^{2.61(98)}$ side chain to have several distinct polar and ionic interactions that mediate different aspects of GnRH receptor function. We have previously identified multiple specific interactions of a single functional group for a serotonin−receptor complex (*2*). However, such a detailed interaction map has proven more difficult to develop for peptide receptors, due to the increased complexity of the ligand structure. We now identify and characterize multiple interactions of a single amino acid side chain of the GnRH receptor by systematic mutagenesis, coordinated ligand modification, and computational modeling, combined with measurement of multiple parameters of receptor function. Our results suggest that two distinct hydrogen bonds of $Asp^{2.61(98)}$ mediate ligand recognition, while its negative charge is required for an intramolecular interaction that regulates both ligand binding and receptor expression.

EXPERIMENTAL PROCEDURES

Peptides. GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-$GlyNH_2$), [D-Ala^6,Pro^9NHEt]-GnRH (GnRH-A), [D-Ala^6]-GnRH, [D-Lys^6]-GnRH, and [Gln^8]-GnRH were from Bachem, King of Prussia, PA; [Gln^1]-GnRH, [Trp^2]-GnRH, [Phe^2]-GnRH, [Tyr^2]-GnRH, [Nal^2]-GnRH, [Trp^2,D-Nal^6]-GnRH, [Leu^3]-GnRH, [His^5,Trp^7,Tyr^8]-GnRH (GnRH II), [D-Trp^6]-GnRH, [HO-Pro^9]-GnRH, and the antagonist Cetrorelix ([Ac-D-Nal^1,D-Cpa^2,D-Pal^3,D-Cit^6,D-Ala^{10}]-GnRH) were prepared by conventional solid-phase synthesis.

Receptor Amino Acid Residue Numbering. As the GnRH receptor is used as a model of the rhodopsin-like GPCR family of receptors, comparison of equivalent amino acid residues in other GPCRs is often necessary. A numbering scheme, in which amino acids of the GnRH receptor are numbered relative to the most conserved residues in the TMS of the rhodopsin-like GPCRs, is used to facilitate comparison with other receptors (*10*). The amino acid identifier, which follows the name of the amino acid, consists of the TMS number followed by the position relative to the most conserved residue in that TMS, which is assigned the number 50, and the sequence number of the amino acid in its receptor, in parentheses. Thus, Asp^{98}, which is located near the cytosolic end of TMS 2 and 11 residues carboxy-terminal to the most conserved locus in this TMS, is designated $Asp^{2.61(98)}$. Mutations are designated by the amino acid identifier preceding the name of the mutant amino acid, e.g., $^{2.61(98)}$Glu for the mutant receptor in which $Asp^{2.61(98)}$ is substituted with Glu.

DNA Constructs, Cell Culture, and Transfection. The mutations $Asp^{2.61(98)}$Glu, $Asp^{2.61(98)}$Asn, $Asp^{2.61(98)}$Ala, and $Asp^{2.61(98)}$Val were introduced into the human GnRH receptor as previously described (*11*) using the Altered Sites Mutagenesis System (Promega, Madison, WI) and subcloned into the pcDNA1/Amp expression vector (Invitrogen, San Diego, CA). Epitope-tagged constructs were prepared for immunoblot analysis of mutant receptor expression. A carboxy-terminal domain derived from a human putative type II GnRH receptor (*12*) and a carboxy-terminal hexa-histidine tag were appended to the carboxy termini of the wild-type and mutant receptors using a combination of polymerase chain reaction and multifragment subcloning into the pcDNA3 expression vector (Invitrogen). All DNA constructs were sequenced, and the presence of mutations and epitope tags was confirmed.

COS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and transfected as previously described, using Lipofectamine (Life Technologies Inc., Gaithersburg, MD) (*6*). Cells were seeded into 12- or 24-well plates the day after transfection, and assays were performed 1 or 2 days later.

Phosphatidylinositol Hydrolysis. Accumulation of inositol phosphates (IP) was determined as described previously (*13*). Transfected cells were plated into multiwell plates and labeled for 16 h in Dulbecco's modified Eagle's medium containing [^{3}H]-*myo*-inositol (0.5 μCi, NEN, North Billerica, MA). After the plates were washed with serum-free medium, the cells were incubated for 45 min at 37 °C with varying concentrations of peptides in the presence of 20 mM LiCl. The incubation was terminated by removal of the medium and addition of 10 mM formic acid. [^{3}H]-IP was separated on Dowex ion-exchange columns and eluted with 1 M ammonium formate in 0.1 M formic acid.

Quantitative Immunoblotting. Cell membranes were prepared from transfected COS-1 cells, solubilized in CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), deglycosylated, electrophoresed, and immunoblotted as previously described (*14*). Bound antibody was detected using the ECL Plus Western Blotting kit (Amersham, Arlington Heights, IL) and quantified using the Storm imaging system (Molecular Dynamics, Sunnyvale, CA). The epitope-tagged wild-type receptor was used to construct a standard curve against which expression of mutant receptors was measured. To maintain constant protein concentrations with different concentrations of the tagged receptor, the deglycosylated, CHAPS-solubilized extract of cells transfected with epitope-tagged wild-type receptor was diluted with appropriate volumes of *N*-glycosidase F-treated CHAPS extract from cells transfected with untagged receptor.

Ligand Binding Assays. GnRH-A and Cetrorelix were radioiodinated using Iodogen (Pierce Chemical Co., Rockford, IL) following published protocols (*15*), except that ^{125}I-Cetrorelix was eluted from the QAE Sephadex column in the presence of acetonitrile (30%) to maintain solubility of the peptide. ^{125}I-Cetrorelix competition binding assays were performed essentially as described for ^{125}I-GnRH-A (*16*) with

minor variations. Transfected cells, in 24-well plates, were incubated for 4 h at 25 °C with ^{125}I-Cetrorelix (200 000 cpm/well) and varying concentrations of unlabeled peptides in a total volume of 0.4 mL per well of HEPES-buffered Krebs−Ringer solution (pH 7.4) with 1% BSA. The incubation was terminated by removal of the medium, and bound radioactivity was collected in 1 M NaOH after the cells were washed 3 times with cold buffer containing 0.2% BSA.

Data Analysis. IC_{50} (peptide concentration required for 50% inhibition of ^{125}I-Cetrorelix binding) values were estimated using nonlinear curve-fitting (KaleidaGraph, Synergy Software) (*13*). EC_{50} (agonist concentration required for half-maximal response) values for IP production were calculated using KaleidaGraph or Prism (Graphpad, San Diego, CA), and figures were prepared using Prism.

Computational Methods. A manual iterative approach was used to develop a model of the GnRH−receptor complex as described elsewhere.[2] The [Trp^2]-GnRH−receptor complex was constructed from the GnRH−receptor complex by mutation of His^2 in GnRH and energy minimization of the resultant complex.

RESULTS

Mutation of Asp$^{2.61(98)}$ Decreases GnRH-Stimulated IP Accumulation and Receptor Expression. The Asp$^{2.61(98)}$ residue of the human GnRH receptor was mutated to Glu, Asn, Ala, and Val in order to analyze the roles of charge, size, and polarity in its function. While all mutant receptors mediated GnRH-stimulated IP accumulation in transiently transfected COS-1 cells, all of the mutations altered receptor function (Figure 1). The E_{max} for GnRH-stimulated IP accumulation in the conservatively substituted, $^{2.61(98)}$Glu mutant was slightly lower than in the wild-type GnRH receptor, while E_{max} values for the $^{2.61(98)}$Asn, $^{2.61(98)}$Ala, and $^{2.61(98)}$Val mutants were markedly decreased (Figure 1, Table 1). None of the mutant receptors exhibited measurable binding of ^{125}I-GnRH-A, preventing analysis of expression levels by conventional ligand binding assays. Therefore, a quantitative immunoblotting assay of epitope-tagged receptors was developed to assess expression of the mutant receptors. EC_{50}s of epitope-tagged wild-type and mutant receptors were comparable with their untagged counterparts (Table 1). E_{max} values were increased for all epitope-tagged receptors (Table 1), consistent with increased expression of the tagged receptors due to the addition of the carboxy-terminal domain (*14*). With the exception of the $^{2.61(98)}$Asn constructs, the E_{max} values of the unmodified and epitope-tagged receptors correlate closely. It is not known why the $^{2.61(98)}$Asn construct shows a relatively large increase in E_{max} in comparison with the other mutants on addition of the carboxy-terminal tail and epitope tag. Serial dilution of the epitope-tagged wild-type GnRH receptor showed that there is a linear relationship between epitope-tagged receptor concentration and chemiluminescence on immunoblots (data not shown). Comparison of the epitope-tagged mutant receptors with epitope-tagged wild-type receptor dilution curves (see Experimental Procedures) on the same immunoblot showed decreased expression of the mutant constructs (Figure 1, Table 1). The receptors measured by this technique include proteins located both on interior cell membranes and on the cell surface and may therefore not precisely correlate with the number of receptors able to interact with GnRH in IP asssays. However, the decreased expression of the mutant receptors suggests that the decreased E_{max} values obtained with the mutants may result, in part, from decreased receptor expression.

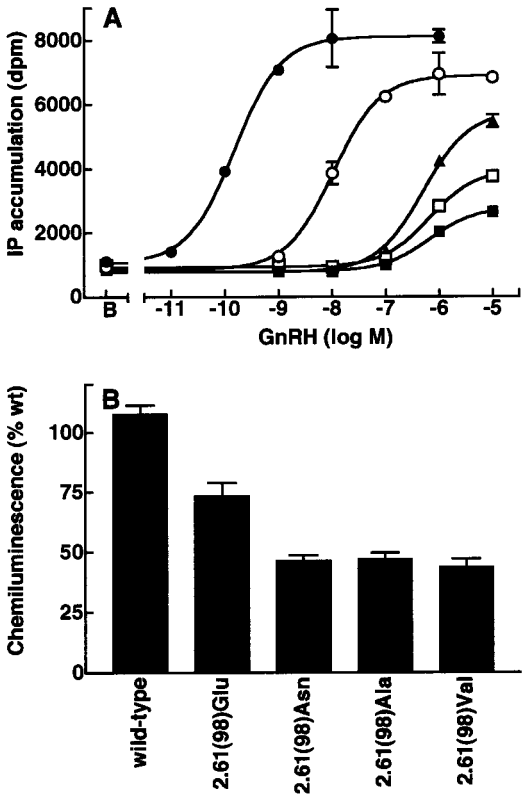

FIGURE 1: Decreased E_{max} and decreased expression of mutant receptors. GnRH-stimulated IP accumulation (A) was measured in COS-1 cells transfected with wild-type (●) and $^{2.61(98)}$Glu (○), $^{2.61(98)}$Asn (■), $^{2.61(98)}$Ala (□), or $^{2.61(98)}$Val (▲) mutant GnRH receptor constructs. Data are the mean ± SE of a representative experiment performed in triplicate. For quantitative immunoblot analysis of receptor protein expression, cell membranes from COS-1 cells transfected with epitope-tagged wild-type and mutant receptor constructs were CHAPS-solubilized and deglycosylated before gel electrophoresis and immunoblot detection. A standard curve was generated by dilution of epitope-tagged wild-type GnRH receptor as described under Experimental Procedures. Expression of mutant receptors (B) was estimated using a standard curve on the same blot. Data are the mean ± SE of 6 determinations in 3 separate experiments.

Mutation of Asp$^{2.61(98)}$ Increases EC_{50} of GnRH. Mutation of Asp$^{2.61(98)}$ increased the EC_{50} values for GnRH-stimulated IP accumulation in all mutant receptors (Figure 1, Table 1). We have previously demonstrated that there is little receptor reserve for the human GnRH receptor in this transfection system and, therefore, increased EC_{50} values reflect decreased agonist affinity (*6*). The Asp$^{2.61(98)}$Glu mutant, which preserves the carboxyl side chain of the wild-type Asp, exhibited a 116-fold increase in EC_{50} (35.7 ± 7.2 nM) compared with the wild-type receptor (EC_{50} 0.31 ± 0.06 nM). The increased EC_{50} found with the conservative $^{2.61(98)}$Glu substitution shows that the position of the carboxyl group relative to the peptide backbone of the receptor is critical for high-affinity binding of GnRH. The EC_{50}s for mutant receptors with uncharged side chains, Asn, Ala, and Val, in position 2.61-

[2] Konvicka, Sealfon, Weinstein, and Guarnieri, in preparation.

Table 1: GnRH-Stimulated IP Accumulation and Immunoblot Analysis of Receptor Expression[a]

receptor construct	IP accumulation		immunoblot detection
	EC_{50} (nM)	E_{max} (% wild type)	% tagged wild type[b]
wild-type ($Asp^{2.61(98)}$)	0.22 ± 0.04 (3)	100	
$^{2.61(98)}$Glu	18.9 ± 4.5 (3)	84.4 ± 10.9 (3)	
$^{2.61(98)}$Asn	2073 ± 709 (4)	17.3± 4.2 (4)	
$^{2.61(98)}$Ala	1712 ± 741 (3)	28.6 ± 7.9 (3)	
$^{2.61(98)}$Val	3239 ± 1382 (3)	66.3 ± 8.0 (3)	
tagged wild type[a]	0.28 ± 0.11 (3)	289 ± 48 (3)	107.6 ± 3.5 (6)
tagged $^{2.61(98)}$Glu	17.3 ± 2.8 (3)	218 ± 38 (3)	73.3 ± 5.5 (6)
tagged $^{2.61(98)}$Asn	3217 ± 119 (3)	126 ± 36 (3)	46.3 ± 2.3 (6)
tagged $^{2.61(98)}$Ala	2530 ± 438 (3)	71 ± 17 (3)	47.0 ± 2.6 (6)
tagged $^{2.61(98)}$Val	2845 ± 482 (3)	155 ± 24 (3)	43.5 ± 3.5 (6)

[a] IP accumulation in response to GnRH was determined in triplicate in COS-1 cells transfected with wild-type and mutant receptor constructs. Numbers of experiments are indicated in parentheses. Immunoblot analysis was performed on COS-1 cells transfected with epitope-tagged receptor constructs as described under Experimental Procedures. [b] Epitope-tagged constructs have a carboxy-terminal domain and carboxy-terminal hexa-histidine sequence as described under Experimental Procedures.

(98) were further increased, 3841-, 7782- and 14 722-fold, respectively (Figure 1, Table 1). The decreased affinity of the Asn mutant shows that the isosteric amide side chain of Asn cannot substitute for the carboxyl group of the Asp residue. This suggests that a negatively charged carboxylate (rather than an uncharged carboxylic acid) side chain is required for high-affinity interaction with GnRH, since the −NH of Asn can substitute in hydrogen bonds for the −OH of aspartic acid, but not for the $-O^-$ of aspartate. The decreased affinity of the mutant receptors could result from disruption of a direct interaction of the $Asp^{2.61(98)}$ side chain with a functional group on the ligand, or from disruption of protein folding which has indirect effects on ligand binding. The small responses obtained with the $^{2.61(98)}$Ala and $^{2.61(98)}$Asn mutant receptors (28.6% and 17.3% of wild-type) make it difficult to perform detailed pharmacological experiments. Consequently, further experiments to determine the role of the $Asp^{2.61(98)}$ side chain in ligand binding focused on the $^{2.61(98)}$Glu and $^{2.61(98)}$Val mutants.

Pharmacophore Profiling: Position 2-Substituted Analogues Are Less Sensitive to $Asp^{2.61(98)}$ Mutations. A series of GnRH analogues with substitutions in different positions along the length of the peptide was used to assess whether the decreased affinity of the $^{2.61(98)}$Glu and $^{2.61(98)}$Val mutant receptors for GnRH resulted from loss of an interaction with a particular amino acid side chain in the GnRH molecule. The ratios of EC_{50} for the $^{2.61(98)}$Glu and $^{2.61(98)}$Val mutations relative to wild-type [$EC_{50(Glu/wt)}$ and $EC_{50(Val/wt)}$, Table 2] quantify the decreases in affinity resulting from the $Asp^{2.61(98)}$Glu and $Asp^{2.61(98)}$Val mutations, respectively. Peptides with substitutions in positions 1, 3, 5, 6, 7, 8, 9, and 10 exhibited characteristics similar to those of unmodified GnRH (Figure 2, Table 2). EC_{50} values for peptide-stimulated IP accumulation were increased by 2 orders of magnitude in the $^{2.61(98)}$Glu mutant and by 3−4 orders of magnitude in the $^{2.61(98)}$Val mutant (Figure 2, Table 2). Notably, [Gln^8]-GnRH, in which the only positive charge in the GnRH peptide (Arg^8) is substituted with an uncharged side chain, also exhibited large increases in EC_{50} at the mutant receptors [$EC_{50(Glu/wt)}$ 177.7 and $EC_{50(Val/wt)}$ > 4600], similar to those for native GnRH. These data suggest that $Asp^{2.61(98)}$ is unlikely to interact with side chains at positions 1, 3, and 5−10 of GnRH, In contrast, [Trp^2]-GnRH exhibited only a 3-fold increase in EC_{50} at the $^{2.61(98)}$Glu mutant. The 200-fold increase in EC_{50} of [Trp^2]-GnRH in the $^{2.61(98)}$Val mutant (Figure 2, Table 2) was also relatively small compared with His^2-containing peptides. Subsequent analysis with a series of position 2-substituted GnRH analogues showed smaller changes in EC_{50}values for the $^{2.61(98)}$Glu and $^{2.61(98)}$Val mutants for all peptides which do not have His in position 2 [$EC_{50(Glu/wt)}$ 1.3−17.6 and $EC_{50(Val/wt)}$ 18.9−2989, Figure 3, Table 2]. These results show that the $Asp^{2.61(98)}$ side chain determines specificity for the His^2 side chain of GnRH.

A series of modified GnRH analogues with aromatic substitutions (Trp, Tyr, Phe, and Nal) in position 2 was used to analyze the interaction of $Asp^{2.61(98)}$ with His^2. All of these analogues had increased EC_{50} values compared with native GnRH, at the wild-type receptor (Figure 3, Table 2). The His side chain differs from the substituted side chains in having a potential hydrogen bond donor group at the δ position. Thus, the decreased affinity of the analogues suggests that the δ NH group of the His side chain contributes to the high-affinity interaction of native GnRH with the wild-type receptor. The $^{2.61(98)}$Glu mutant exhibited a loss of preference for native GnRH over the position 2-substituted analogues (Figure 3, Table 2). This suggests that the mutation disrupts an interaction between $Asp^{2.61(98)}$ and the δ NH group of His^2. However, the decrease in affinity of the substituted analogues, which do not have a δ NH group, for the mutant receptor [$EC_{50(Glu/wt)}$ 1.3−17.6] indicates that the mutation causes an additional perturbation of the ligand−receptor interaction. This inference is also supported by the size of the decrease in affinity of His^2-containing peptides [$EC_{50(Glu/wt)}$ 76−178], which is large for disruption of a single hydrogen bond (*17*).

All peptides exhibited further increased EC_{50}s at the uncharged $^{2.61(98)}$Val mutant receptor compared with the $^{2.61(98)}$Glu mutant (Figure 3, Table 2). The $EC_{50(Val/Glu)}$ ratio, which indicates the increase in EC_{50} between the $^{2.61(98)}$Glu and $^{2.61(98)}$Val mutants, was similar for peptides with and without His^2 (Table 2). This shows that removal of the negative charge has an additional effect on peptide binding that is not related to recognition of the His^2 side chain and suggests that the $Asp^{2.61(98)}$ side chain has an additional, charge-dependent interaction that also contributes to high-affinity agonist binding.

Ligand Binding Confirms Decreased Affinities in $^{2.61(98)}$Glu Mutant. The results of the IP assays, which indicated that mutation of $Asp^{2.61(98)}$ interfered with recognition of the amino terminus of agonists, suggested that binding of antagonists, which have extensive modifications of the amino terminus, would be less sensitive to mutation of $Asp^{2.61(98)}$. The radio-iodinated antagonist ^{125}I-Cetrorelix bound the wild-type and $^{2.61(98)}$Glu mutant receptors with high affinity (Table 3, Figure 4). Specific binding ranged from 1700 to 8000 cpm per tube for the wild-type and between 4200 and 8500 cpm per tube for the $^{2.61(98)}$Glu mutant. Nonspecific binding varied between 1000 and 5500 cpm per tube. The $^{2.61(98)}$Val, $^{2.61(98)}$Ala, and $^{2.61(98)}$Asn mutant receptors showed a small amount of displaceable binding of ^{125}I-Cetrorelix (~1000 cpm/well,

Table 2: Summary of IP Accumulation Stimulated by GnRH Analogues with Various Side Chain Substitutions[a]

peptide	wild-type EC_{50} (nM)	$^{2.61(98)}$Glu mutant		$^{2.61(98)}$Val mutant		
		EC_{50} (nM)	$EC_{50(Glu/wt)}$	EC_{50} (nM)	$EC_{50(Val/wt)}$	$EC_{50(Val/Glu)}$
GnRH	0.31 ± 0.06 (15)	35.7 ± 7.2 (14)	116.2	3176 ± 634 (11)	10340	89.0
[Gln^8]-GnRH	2.35 ± 0.55 (4)	417 ± 117 (4)	177.7	>10000 (4)	>4600	>27
[Gln^1]-GnRH	1.6 (1)	121 (1)	75.6	5652 (1)	3533	46.7
[Leu^3]-GnRH	392 ± 281 (3)	31415 ± 1785 (2)	80.1	>10000 (2)	>25	
GnRH II ([His^5,Trp7,-Tyr^8]-GnRH)	2.4 ± 0.46 (4)	366 ± 51 (3)	152.4	>10000 (3)	>4100	>27
GnRH-A (des Gly^{10}, [D-Ala^6,Pro^{9}NHEt]-GnRH)	0.22 ± 0.05 (4)	17.6 ± 7.0 (4)	81.6	926 ± 112 (3)	4305	52.7
[HO-Pro^9]-GnRH	1.90 ± 0.48 (3)	322 ±+ 141 (2)	169.3	>10000 (2)	>5263	>31.1
[Trp^2]-GnRH	1.13 ± 0.09 (3)	3.4 ± 1.3 (3)	3.0	226 ± 85 (3)	199	67.0
[Phe^2]-GnRH	5.73 ± 0.82 (3)	39.7 ± 7.8 (3)	6.9	17135 ± 10865 (2)	2989	432
[Tyr^2]-GnRH	2.22 ± 0.48 (4)	39.2 ± 9.8 (3)	17.6	6167 ± 1458 (3)	2773	157.3
[Nal^2]-GnR^H	16.3 ± 3.8 (5)	281 ± 111 (4)	17.3	>10000 (3)	>666	>28
[Trp^2,D-Nal^6]-GnRH	0.28 ± 0.15 (5)	0.37 ± 0.12 (5)	1.3	5.3 ± 0.9 (6)	18.9	14.3

[a] IP accumulation was measured in COS-1 cells which were transiently transfected with wild-type or mutant GnRH receptor constructs, and treated with a library of GnRH analogues. Data are the mean ± SE of the number of experiments indicated in parentheses. $EC_{50(Glu/wt)}$ and $EC_{50(Val/wt)}$ are the ratios of the EC_{50} for the mutant receptor to the EC_{50} for the wild-type receptor and indicate the decrease in peptide potency associated with the mutation. $EC_{50(Val/Glu)}$ is the ratio of the EC_{50} for the $^{2.61(98)}$Glu mutant receptor to the EC_{50} for the $^{2.61(98)}$Val mutant receptor and indicates the loss of function due to removal of the negative charge from the 2.61(98) locus, as distinct from the disruption of interactions that are revealed by the charge-conserving $^{2.61(98)}$Glu mutation.

not shown) which was too low to allow detailed pharmacological studies. This low total binding was probably due to the decreased expression of the mutant receptors (Figure 1, Table 1) combined with decreased affinity for ^{125}I-Cetrorelix.

IC_{50} values for binding of GnRH analogues (Table 3) confirmed that the increased EC_{50} values for IP accumulation in the $^{2.61(98)}$Glu mutant receptor (Table 2) are the consequence of decreased affinity. IC_{50} values for binding of GnRH and analogues with His in position 2 were increased 25−350-fold in the $^{2.61(98)}$Glu mutant compared with the wild-type receptor, while analogues with substitutions in position 2 showed only slightly decreased affinity for the $^{2.61(98)}$Glu mutant (Figure 4, Table 3).

Computational Modeling. Molecular modeling of the GnRH receptor−ligand complexes yielded equilibrated structures of agonist peptides bound to the human GnRH receptor (Figure 5). The equilibrated complex of GnRH with the receptor was stabilized by hydrogen bond and hydrophobic interactions.[2] Asp$^{2.61(98)}$ formed two hydrogen bonds, one to the δ NH group of the His^2 side chain and one to the backbone NH group of the Trp^3 residue of the ligand. The Asp$^{2.61(98)}$ side chain also formed an intramolecular charged hydrogen bond with the Lys$^{3.32(121)}$ residue in TMS 3. The Lys$^{3.32(121)}$ side chain, in turn, hydrogen bonded the backbone C═O group of the Ser^4 residue of the ligand (Figure 5A). The equilibrated complex of [Trp^2]-GnRH with the receptor showed the same interactions as native GnRH, except that there was no hydrogen bond of Asp$^{2.61(98)}$ with the side chain of the Trp residue that was substituted for the native His^2 (Figure 5B).

DISCUSSION

Decreased ligand binding affinity in mutant receptors can have different structural origins. Mutagenesis may disrupt direct interactions of the receptor side chain with side chain functional groups of peptide ligands, or with functional groups on the peptide backbone of the ligand. It can also disrupt intramolecular bonds within the receptor. Disruption of the intramolecular interactions which constrain the conformation of the peptide backbone of the receptor can alter interactions which involve functional groups that are distant from the mutated residue. Mutation of a residue such as Asp, of which the side chain potentially forms up to four noncovalent bonds at the same time, can simultaneously modify both direct and indirect interactions with the ligand. Altering ligand structure in parallel with mutating the receptor can delineate the functional groups of the ligand that are involved in interactions and help distinguish direct receptor−ligand interactions from indirect structural effects (*4*). A library of GnRH analogues, consisting of peptides with substitutions for most of the side chains of GnRH, was used to determine whether the decreased affinity for GnRH after mutation of Asp$^{2.61(98)}$ resulted from disruption of a specific side chain interaction. Most side chain substitutions decreased ligand affinity for the wild-type receptor (Table 2) (*1*, *18*). The decreased affinity can result from steric interference, changes in peptide conformation (*19*), or disruption of a side chain interaction with the receptor (*5*, *7*). Our studies indicate that the Asp$^{2.61(98)}$ residue of the GnRH receptor contributes to high-affinity agonist binding both directly, via interactions with the ligand, and indirectly, via intramolecular interactions that contribute to formation of the binding pocket.

Asp$^{2.61(98)}$ Does Not Form Ionic Interactions with the Ligand. The negative charge of the Asp$^{2.61(98)}$ side chain could interact with a positive charge on the ligand. At physiological pH, there is only one positively charged functional group (the Arg^8 side chain) in the GnRH peptide. Arg^8 is required for high-affinity binding of GnRH to mammalian GnRH receptors (*1*, *18*, *20*), and substitution of Arg^8 with uncharged Gln or Tyr (in GnRH II) residues decreases the affinity for the receptor (Figure 2, Table 2) (*1*). The position 8-substituted ligands had decreased affinity for the $^{2.61(98)}$Glu and $^{2.61(98)}$Val mutant receptors compared with the wild-type receptor, and they also maintained lower affinity than native GnRH in the mutant receptors. The comparable decrease in affinity of peptides with and without Arg in position 8 indicates that the Asp$^{2.61(98)}$ side chain does not interact with

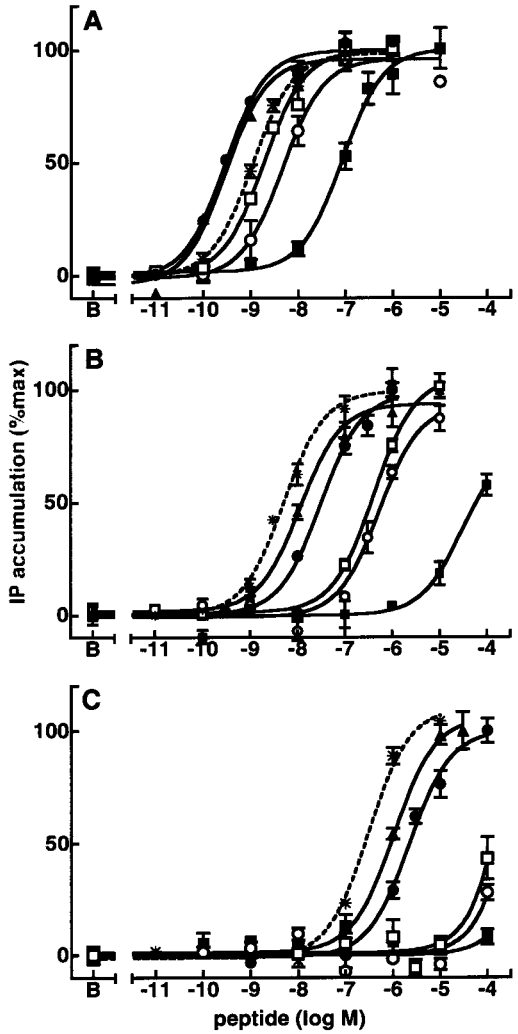

FIGURE 2: IP accumulation stimulated by GnRH analogues in wild-type and mutant GnRH receptors. COS-1 cells transfected with wild-type (A) or mutant $^{2.61(98)}$Glu (B) and $^{2.61(98)}$Val (C) GnRH receptor constructs were treated with increasing concentrations of GnRH (●), [Trp^2]-GnRH (*), [Leu^3]-GnRH (■), GnRH II ([His^5,Trp^7,-Tyr^8]-GnRH) (□), [Gln^8]-GnRH (○), or GnRH-A (▲). Data are the mean ± SE of representative experiments performed in triplicate and expressed as percent of GnRH-stimulated E_{max} in the same experiment. The number of experiments performed with each peptide is shown in Table 2.

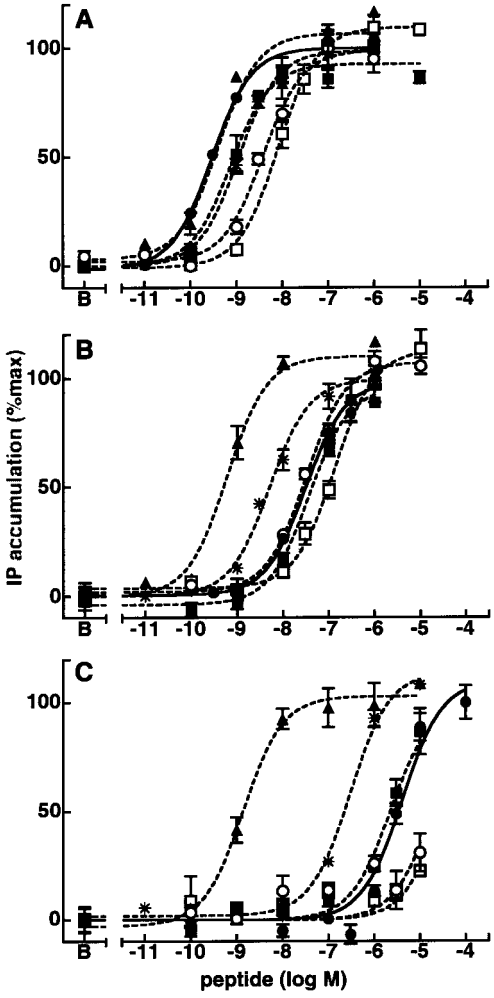

FIGURE 3: IP accumulation stimulated by His^2-substituted analogues of GnRH. COS-1 cells transfected with wild-type (A) or mutant $^{2.61(98)}$Glu (B) and $^{2.61(98)}$Val (C) GnRH receptor constructs were stimulated with GnRH (●) or a series of position 2-substituted GnRH analogues, [Trp^2]-GnRH (*), [Phe^2]-GnRH (○), [Tyr^2]-GnRH (■), [Nal^2]-GnRH (□), or [Trp^2,D-Nal^6]-GnRH (▲). Data are mean ± SE of representative experiments performed in triplicate and expressed as percent of GnRH-stimulated E_{max} in the same experiment. The number of experiments performed with each peptide is shown in Table 2.

the Arg^8 side chain. The data are consistent with our previous identification of Glu$^{7.32(301)}$, in the third extracellular loop of the mouse GnRH receptor, as the residue that determines high-affinity binding of GnRH through an interaction that involves the Arg^8 side chain (*7*).

The present work supports an interaction of His^2 with the Asp$^{2.61(98)}$ side chain of the receptor, and suggests that the His side chain is unprotonated when GnRH first binds to the receptor. The His^2 side chain in GnRH has a p*K* of 6.0 (*21*, *22*). Optimal binding to the GnRH receptor occurs at pH 7.0−7.5, where the His^2 side chain is predominantly uncharged. At pH 6.0, where the His^2 side chain is 50% protonated, binding is decreased to 50% (*23*). Furthermore, the Asp$^{2.61(98)}$Glu mutation, which specifically decreases the affinity for His^2-containing peptides, conserves the negative charge of the Asp$^{2.61(98)}$ side chain. Therefore, it is unlikely that an ionic interaction occurs between the Asp$^{2.61(98)}$ and His^2 side chains.

Table 3: Summary of Competition Binding Experiments[a]

peptide	IC_{50} (nM) wild type	$^{2.61(98)}$Glu mutant	affinity loss
GnRH	1.4 ± 0.2 (4)	490 ± 40 (5)	350
[D-Trp^6]-GnRH	0.6 ± 0.2 (3)	15 ± 3 (4)	25
[D-Ala^6]-GnRH	2.6 ± 0.6 (3)	118 ± 12 (3)	45
[D-Lys^6]-GnRH	7 ± 1 (2)	950 ± 600 (2)	136
GnRH-A	0.8 ± 0.2 (2)	160 ± 10 (2)	200
[Trp^2]-GnRH	4.4 ± 0.3 (4)	17 ± 2 (5)	3.9
[Phe^2]-GnRH	600 ± 390 (2)	1123 ± 277 (2)	1.9
[Trp^2, D-Nal^6]-GnRH	4.1 ± 0.5 (2)	4.4 ± 0.7 (2)	1.1
Cetrorelix	0.61 ± 0.09 (5)	1.63 ± 0.04 (3)	2.7

[a] Competition binding assays were performed on COS-1 cells transfected with wild-type or mutant GnRH receptors using ^{125}I-Cetrorelix and various concentrations of unlabeled peptides as described under Experimental Procedures. Data are the mean ± SE of the indicated number of experiments performed in triplicate. The affinity loss for each peptide is calculated as the ratio of the IC_{50} in the mutant receptor to the IC_{50} in the wild-type receptor.

Asp$^{2.61(98)}$ Has Distinct Roles in Ligand Binding: Recognition of His^2 and Backbone Effects. In addition to its negative

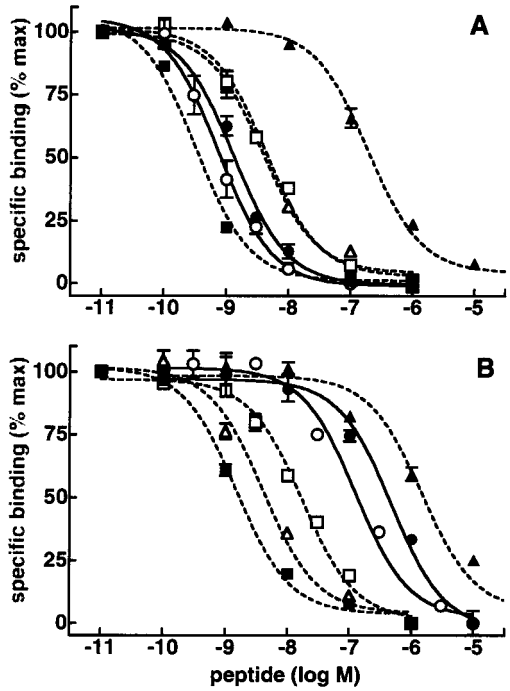

FIGURE 4: Antagonist competition binding assays in wild-type and $^{2.61(98)}$Glu mutant receptors. COS-1 cells transfected with wild-type (A) or mutant $^{2.61(98)}$Glu (B) GnRH receptor constructs were incubated with ^{125}I-Cetrorelix (a GnRH antagonist) and the indicated concentrations of GnRH (●), GnRH-A (○), Cetrorelix (■), [Trp^2]-GnRH (□), [Phe^2]-GnRH (▲) or [Trp^2,D-Nal^6]-GnRH (△). Peptides with substitutions for His^2 are indicated with dashed lines. Data are mean ± SE of representative experiments performed 2−5 times (Table 3) in triplicate.

charge, the Asp side chain has two oxygen atoms which can act as hydrogen bond acceptors. The individual roles of the ionic and hydrogen bonding properties of the Asp$^{2.61(98)}$ side chain in receptor function were assessed by mutating the Asp$^{2.61(98)}$ residue to Glu, Asn, Ala, and Val. All of the mutations disrupted receptor function, but the degree of disruption varied. Increasing the side chain length while retaining the carboxyl group, in the $^{2.61(98)}$Glu mutant, decreased the affinity for GnRH more than 100-fold. Removal of the potential negative charge, in the Asn, Ala, and Val mutants, had a still greater effect. This graded effect suggests two distinct functional roles for the Asp$^{2.61(98)}$ side chain: one function related to the position of the carboxyl group, as revealed by the $^{2.61(98)}$Glu mutant, and a second function related to its negative charge, as revealed by the mutants with uncharged substitutions in position 2.61(98). A library of substituted GnRH analogues showed that Asp$^{2.61(98)}$ determines specificity for the His^2 side chain of GnRH. An unprotonated His side chain potentially has a hydrogen bond donor group, which could form hydrogen bonds with the oxygen atoms of the Asp$^{2.61(98)}$ side chain, in either the δ or the ϵ position. Substitution of His with Phe removes both potential proton donors, while substitution with Trp preserves an NH group in the ϵ position and substitution with Tyr generates an OH group in the η position (*16*). Peptides in which His^2 was substituted with Trp, Tyr, or Phe had 3.7-, 7.1-, and 20-fold increased EC_{50} values, respectively, at the wild-type receptor. This decrease in affinity is consistent with disruption of a single hydrogen bond (*17*). The decreased affinity of the wild-type receptor for all of the position 2-substituted peptides, including [Trp^2]-GnRH,

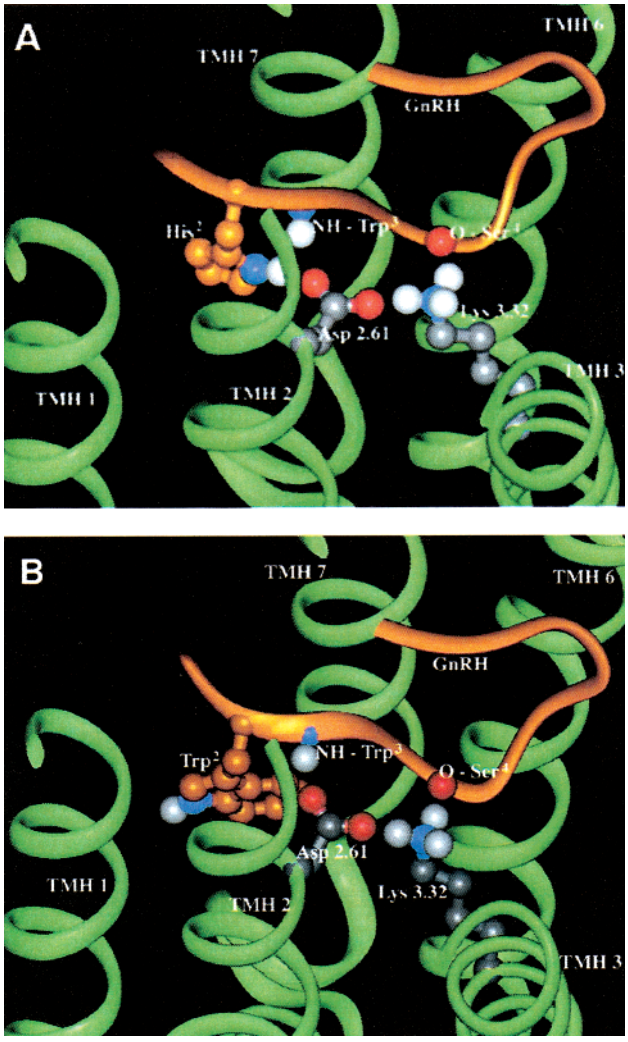

FIGURE 5: Computational models of GnRH receptor−ligand complex. The binding of GnRH (A) and [Trp^2]-GnRH (B) to the wild-type GnRH receptor was modeled as described under Experimental Procedures. The transmembrane helices are represented by a green ribbon, and the ligand backbone is brown. Atoms of key functional groups discussed in the text are rendered according to the atomic color convention: oxygen in red, nitrogen in blue, and hydrogen in white.

which has an ϵ NH group, suggests that the δ NH group of the His^2 side chain underlies the higher affinity of native GnRH for the wild-type receptor.

The $^{2.61(98)}$Glu mutant receptor showed a small decrease in affinity for the position 2-substituted peptides, but showed a larger decrease in affinity for native GnRH, losing the preference for the His^2-containing peptide over the Trp-, Tyr-, and Phe-substituted analogues. The loss of preference for His^2 over other aromatic side chains suggests that the increased side chain length at the 2.61(98) locus in the Glu mutant may not provide the proper geometry for the proposed interaction with the δ NH group of His^2 in native GnRH. However, the decreased affinity of all peptides at the mutant receptor [$EC_{50(Glu/wt)}$ 76−178 for His^2-containing analogues and 1.3−17.6 for peptides without His^2] raises the possibility that Asp$^{2.61(98)}$ forms an additional interaction with GnRH. Interestingly, the computational model does identify a second interaction between Asp$^{2.61(98)}$ and GnRH involving the backbone NH group of Trp^3.

All peptides exhibited lower affinity for the uncharged $^{2.61(98)}$Val mutant receptor than for the $^{2.61(98)}$Glu mutant. These data suggest that the negative charge retains a function in the $^{2.61(98)}$Glu mutant that is lost in the $^{2.61(98)}$Val mutant. To assess the effect of removing the negative charge of the Asp side chain independently of the disruption of the proposed Asp$^{2.61(98)}$− His^2 interaction, it is useful to compare the loss of affinity between the $^{2.61(98)}$Glu mutant and the $^{2.61(98)}$Val mutant [$EC_{50(Val/Glu)}$ in Table 2]. The increase in EC_{50} values was 47−89-fold for His^2-containing peptides and 15−432-fold for His^2-substituted peptides. The lack of difference between peptides that have His^2 and some that do not shows that the negative charge of the Asp$^{2.61(98)}$ side chain does not have a role in recognizing the His^2 side chain, except insofar as it enhances hydrogen bond strength. Comparison of peptides with substitutions for side chains other than His^2 did not reveal any other side chain in the ligand that might depend on the negative charge of the Asp$^{2.61(98)}$ side chain (see Table 2). This suggests either that the charge of Asp$^{2.61(98)}$ is required for an interaction with one or more functional groups that are common to all of the peptides tested, such as functional groups on the peptide backbone, or that it has an indirect effect on the conformation of the ligand binding pocket.

Computational Model Supports Multiple Distinct Roles of Asp$^{2.61(98)}$ Side Chain in Ligand Binding. An initial position of the δ NH group of the His^2 side chain of GnRH near the Asp$^{2.61(98)}$ side chain of the receptor was incorporated into our computational model of GnRH binding to its receptor.[2] The resulting network of hydrogen bonds of Asp$^{2.61(98)}$ in the computational model clarifies the complex changes in receptor function that result from experimental modification of the Asp$^{2.61(98)}$ and His^2 side chains. The equilibrated model (Figure 5A) maintains the initial hydrogen bond between the δ NH group of the His^2 side chain and the Asp$^{2.61(98)}$ side chain. The 3.7−20-fold increase in EC_{50} in the wild-type receptor of position 2-substituted peptides is consistent with loss of this hydrogen bond (*17*). Consistent with the experimental results, the molecular model of [Trp^2]-GnRH binding to the receptor shows no hydrogen bond between Asp$^{2.61(98)}$ and the side chain of Trp^2. However, models of both native GnRH and [Trp^2]-GnRH binding to the receptor show a second hydrogen bond between Asp$^{2.61(98)}$ and the backbone NH group of the residue in position 3 of the ligand. The His^2-substituted peptides form only a single hydrogen bond with Asp$^{2.61(98)}$ in the wild-type receptor. This interaction, with the backbone of the His^2-substituted peptides, is lost when Asp$^{2.61(98)}$ is mutated. These modeling results provide an explanation for our observation that His^2-substituted ligands show a smaller loss of affinity for the $^{2.61(98)}$Glu mutant than His^2-containing ligands.

The ionic interaction of the Asp$^{2.61(98)}$ side chain with the Lys$^{3.32(121)}$ side chain that emerged in the computational model provides a potential explanation for the large increase in EC_{50} of all peptides in the $^{2.61(98)}$Val mutant compared with the $^{2.61(98)}$Glu mutant. In the model, the negative charge at the 2.61(98) locus positions the Lys$^{3.32(121)}$ side chain to form a hydrogen bond with the backbone C═O group of Ser^4 in the ligand. This functional group is common to all of the ligands tested. The proposed interhelical interaction may have an additional role in maintaining the conformation of the ligand binding pocket and constraining the peptide backbone of the receptor. As previously reported, an uncharged substitution for Lys$^{3.32(121)}$ has a phenotype similar to that now reported with mutation of Asp$^{2.61(98)}$, an observation which supports their proposed interaction (*6*).

In conclusion, we have shown that the side chain of the Asp$^{2.61(98)}$ residue of the GnRH receptor has several distinct intermolecular and intramolecular interactions which affect different aspects of agonist binding and receptor expression. Site-directed mutagenesis and ligand modification suggest that Asp$^{2.61(98)}$ selectively binds the δ NH group of the His^2 side chain of the ligand. The Asp$^{2.61(98)}$ side chain forms a second hydrogen bond which is not selective for a specific ligand side chain and which computational modeling identifies as involving a backbone NH group of the ligand. Uncharged substitutions for Asp$^{2.61(98)}$ show that the negative charge of the carboxylate side chain is also required for ligand binding, but its role may be indirect through an intramolecular interaction with the Lys$^{3.32(121)}$ side chain, which is proposed to position the Lys$^{3.32(121)}$ side chain to hydrogen bond a backbone carbonyl group of the ligand. These findings and inferences are consistent with all experimental data on the binding pocket and support a refined model of the molecular basis of GnRH−receptor interaction.

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